MASS SPECTROMETRY OF PROSTAGLANDINS

RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 60/261,577, "Mass Spectrometry of Prostaglandins," filed January 12, 2001, which is herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0002] The present invention relates generally to the analysis of chemical mixtures. More particularly, it relates to a liquid chromatography-electrospray ionization mass spectrometry method for separating and identifying prostaglandin isomers.

BACKGROUND OF THE INVENTION

[0003] Prostaglandins are biologically important metabolites derived from arachidonic acid. FIG. 1 shows schematically the biochemical pathways of arachidonic acid metabolism, indicating the position of the various prostaglandins (PG). As exemplified by the two structures in FIGS. 2A and 2B, prostaglandins are 20-carbon fatty acids that contain a 5-carbon ring. While structurally similar, the molecules are functionally quite diverse. Prostaglandins act as mediators in a large number of physiological processes, including hemostasis and thrombosis, and contribute to pathologic processes associated with inflammation, atherosclerosis, and bronchoconstriction. There is therefore a great deal of interest in elucidating their roles, a process that requires sensitive and specific detection at nanomolar levels in complex biological matrices.

[0004] Gas chromatography-mass spectrometry (GC-MS) has been the traditional tool for detecting metabolites in the arachidonic acid pathway. However, these methods require extensive sample preparation and cumbersome derivatization procedures. Several analytical steps are required for extraction, separation, and purification before derivatization and separation by GC-MS. While these techniques have been improved in recent years, they remain costly and laborious and yield variable results. In addition, arachidonyl-derived lipids in biological fluids, particularly plasma, are known to be relatively unstable and undergo a

variety of transformations when subjected to harsh derivatization conditions. The samples therefore need to be treated carefully, and antioxidants are commonly used to prevent further oxidation.

[0005] Recently, liquid chromatographic techniques have been developed to separate prostaglandin-containing mixtures with minimal sample preparation prior to analysis. When combined with electrospray ionization (ESI) mass spectrometry, LC has picogram detection limits, which is sufficient bioanalytical sensitivity for many applications. Furthermore, MS and tandem MS can often provide necessary structure elucidation to resolve co-eluting species without tedious derivatization and sample manipulation. For example, a method for high performance liquid chromatography/tandem mass spectrometry of F_2-isoprostanes is disclosed in H. Li et al., "Quantitative high performance liquid chromatography/tandem mass spectrometric analysis of the four classes of F_2-isoprostanes in human urine," *Proc. Natl. Acad. Sci.* 96, 1999: 13381-13386. While this method is useful for the particular species studied, it cannot be generalized to all prostaglandins. One of the challenges in combining LC and ESI-MS for analyzing prostaglandins is that optimal conditions for one technique are often not ideal for the other. That is, conditions that maximize ionization efficiencies reduce chromatographic separation resolution, while ideal chromatographic conditions lead to poor electrospray ionization efficiencies.

[0006] This problem is particularly pronounced for the two prostaglandin isomers illustrated in FIGS. 2A and 2B. Prostaglandin D_2 (PGD_2) and prostaglandin E_2 (PGE_2) are isomers having different roles in inflammatory processes. PGD_2 is the major eicosanoid product of mast cells and is released during allergic or asthmatic anaphylaxis, while PGE_2 activates inflammatory processes and is important in fertility and gastric mucosal integrity. Because of these different functions, it is desirable for researchers to be able to distinguish and quantify the two isomers by LC-MS. For sufficient ionization of the two species, particularly at low concentrations or small sample size, negative ion mode is required, which entails basic solution conditions. Under these conditions, however, the species tend to co-elute from the chromatographic column. Because the two prostaglandin structures are so similar, differing only in the reversed positions of a hydroxyl and carbonyl group, their mass spectra cannot distinguish the co-eluted species. Furthermore, while it is often common to distinguish isomers

by their tandem mass spectra (further fragmentation of the parent and subsequent ions), MS^2 and MS^3 tandem mass spectra of the two species are also virtually identical.

[0007] There is a need, therefore, for a LC-MS method for detecting and distinguishing between prostaglandin isomers at low concentrations. It is desirable that the method require little sample preparation and no sample derivatization and be able to detect and distinguish between picogram quantities of different prostaglandins.

SUMMARY OF THE INVENTION

[0008] The present invention provides a method for performing liquid chromatography-mass spectrometry on a chemical mixture containing at least two different prostaglandins. The method allows the two species to be separated and identified at very low concentrations, such as nanomolar. The method is performed by conducting a liquid chromatographic separation of the mixture, adding a basic liquid in sheath flow to the eluent, and performing mass spectrometry, preferably using electrospray ionization, on the diluted eluent. The chromatographic separation is performed under acidic conditions to ensure separation of the prostaglandin species, which are preferably PGD_2 and PDE_2. The basic conditions of the ionization provide for high ionization efficiency in the negative ion mode. Preferably, the mass spectrometry includes MS^4, which allows the two isomers to be distinguished.

[0009] The present invention also provides a method for distinguishing between at least two prostaglandin isomers, such as PGD_2 and PGE_2, using tandem mass spectrometry, preferably MS^4. The spectra are acquired at different ionization energies, at least one of which is capable of producing spectra that are significantly different from each other. Preferably, the ionization energy is varied until an energy is found at which the spectra are most different from each other, thereby facilitating correct identification of the two prostaglandin isomers.

BRIEF DESCRIPTION OF THE FIGURES

[0010] FIG. 1 (prior art) shows the metabolic pathway of arachidonic acid to form prostaglandins.

[0011] FIGS. 2A and 2B (prior art) show the structures of prostaglandins D_2 and E_2.

[0012] FIG. 3 is a schematic diagram of an interface between a chromatography column and an electrospray needle illustrating sheath flow according to the present invention.

[0013] FIG. 4 is a total ion current chromatogram of a PGD_2 and PGE_2 mixture obtained using the LC-ESI MS instrument of FIG. 3.

[0014] FIG. 5 is a total ion current chromatogram of a human plasma fraction spiked with PGD_2 and PGE_2 obtained using the LC-ESI MS instrument of FIG. 3.

[0015] FIGS. 6A-6C show equipment operation and settings used to obtain the chromatogram of FIG. 5.

[0016] FIGS. 7A and 7B (prior art) are schematic diagrams of the collision-induced dissociation schemes of PGD_2 and PGE_2.

[0017] FIGS. 8A-8C are MS^4 spectra of PGD_2 and PGE_2 at three different ionization energies.

DETAILED DESCRIPTION OF THE INVENTION

[0018] The present invention provides a liquid chromatography (LC)-electrospray ionization (ESI) mass spectrometry (MS) method for separating and detecting different prostaglandin species. In particular, the two isomers PGD_2 and PGE_2 can be separated using methods of the invention. Furthermore, based on their tandem mass spectra, the isomers can be distinguished and the identities of the corresponding chromatographic peaks verified. Thus, according to the invention, isobaric prostaglandins PGD_2 and PGE_2 in biological matrices can be distinguished by capillary LC-MS^4 without extensive sample preparation and tedious derivatization. The detection limits of the method approach nanomolar levels when the mass spectrometer is operated in select ion monitoring mode.

[0019] According to a method of the invention, solution conditions are implemented that optimize both chromatographic separation and ionization efficiency. Optimized ionization efficiency is particularly important when the analyte of interest is at low concentrations or the available sample volume is small. In order to optimize conditions for both chromatography and spectrometry, the chromatography is performed under acidic conditions to enhance separation, while the spectrometry is performed under basic conditions to increase ionization efficiency in the negative ion mode. This is accomplished by introducing a basic solution between the two stages using a basic sheath flow liquid.

[0020] FIG. 3 illustrates a portion **10** of a combined LC/MS instrument of the invention showing the end of a chromatographic column **12** and stainless steel tube **14,** which together form an electrospray ionization needle. An additional solution is introduced into the eluent using the tube **14,** which acts as a sheath surrounding the chromatographic column. For this reason, the additional solution is referred to as being in sheath flow. In a preferred embodiment, the sheath liquid is a basic solution. For example, the sheath liquid can be ammonium hydroxide in a solution of methanol and acetonitrile. In general, the sheath liquid is sufficiently volatile to be used in electrospray ionization and has a pH that provides for an ionization efficiency that is at least sufficient for the prostaglandins to be detected. The specific characteristics of a suitable sheath liquid depend upon features of the particular experiment, including the instrument and sample components. Because the sheath flow is introduced only after the chromatographic separation, it has no effect on the separation, and separation can occur under acidic conditions that are effective for providing a satisfactory resolution of the prostaglandin species. Suitable chromatographic solvents depend upon the particular conditions and should allow for sufficient separation resolution and ionization. For example, the solvents can be mixtures of acetic acid, heptafluorobutyric acid, and acetonitrile.

[0021] FIG. 4 shows a total ion current chromatogram obtained from a LC-ESI MS analysis of a mixture of PGD_2 and PGE_2. In this particular example, the capillary column used was a 0.2 x 150 mm Magic C18 column (Michrom BioResources, Auburn, CA) with 5 μm 200 Å packing. Solvent A was 2% acetonitrile and 98% water containing 0.4% acetic acid and 0.005% heptafluorobutyric acid. Solvent B was 90% acetonitrile and 10% water containing 0.005% heptafluorobutyric acid. Isocratic separation was performed with 26% solvent B at a flow rate of 4 μL/min. The sample concentration was 1 ng/μL and the injection volume 2 μL. The eluent was combined with a sheath liquid of 0.1% NH_4OH in a solution of 50% methanol and 50% acetonitrile. As further confirmed by mass spectrometry (discussed below), the PGD_2 and PGE_2 peaks were well separated. Note that the specific details listed are for illustration purposes only and in no way limit the scope of the invention.

[0022] The LC-MS separation was also performed when the prostaglandins were added to a human plasma fraction from which human serum albumin and proteins with molecular weight greater than 10 kDa were removed. The total ion current (TIC) chromatogram for this

mixture is shown in FIG. 5. In this example, capillary liquid chromatography was performed using a MAGIC 2002 system (Michrom BioResources, Auburn, CA) and mass spectrometry with a Finnigan LCQ Deca (ThermoFinnigan, San Jose, CA). The chromatographic column was a Magic C18 reversed phase silica column (Michrom) with a 5 μm particle size and 200 Å pore size. Relevant instrument settings are shown in FIGS. 6A-6C.

[0023] In this example, the plasma fraction was spiked with PGD_2 and PGE_2 at a concentration of 50 pg/μL of each prostaglandin. 2 μL of this solution was injected and analyzed. Solvents A and B were as described above for FIG. 4. The separation was performed isocratically with 26% solvent B at 4 μL/min with a sheath liquid of 0.1% NH_4OH in a 50:50 mixture of methanol and acetonitrile. As shown in FIG. 5, physiologically relevant concentrations of PGD_2 and PGE_2 can be detected using the method of the invention under these conditions.

[0024] The present invention also includes methods for distinguishing between prostaglandin isomers using tandem mass spectrometry. FIGS. 7A and 7B show collision-induced dissociation schemes for the D_2 and E_2 prostaglandin isomers. Note that the sequential generations of ion fragments for the two species have identical masses. Tandem mass spectrometry performed by the present inventors on both isomers yields a parent ion $[M-H]^-$ at a mass-to-charge ratio (m/z) of 351.2, an MS^2 ion $[M-H-H_2O]^-$ at an m/z of 333.3, an MS^3 ion $[M-H-2H_2O]^-$ at an m/z of 315.2, and an MS^4 ion $[M-H-2H_2O-CO_2]^-$ at an m/z of 271.3. Thus the standard method of obtaining multiple tandem mass spectra to distinguish isomers is not applicable to separating PGD_2 and PGE_2.

[0025] Although the two isomers follow the same overall dissociation scheme, the different ions generated have different relative energies. For a given ionization energy input, therefore, the extent of dissociation is different for the two isomers. Thus according to the present invention, the two isomers can be distinguished based on the different amounts of dissociation at one or more different energy inputs. ESI in the negative ion mode followed by fragmentation of the parent ions in an ion trap to yield MS^2, MS^3, and MS^4 spectra has been used by the present inventors to distinguish the isobaric species in a mixture. The MS^2 spectra and MS^3 spectra were essentially identical for the two species at all ionization energies.

[0026] However, the MS^4 spectra varied at some of the ionization energies. Three different MS^4 spectra for each of the two isomers at three different ionization energies are shown in FIGS. 8A-8C. FIG. 8A shows the spectra for an ionization energy of 25% of the total available instrument energy. Both spectra show a peak at m/z = 315.2 and m/z = 271.3. The relative heights of the two peaks are substantially equivalent in the two spectra, making the spectra virtually indistinguishable and not useful for identifying the prostaglandin isomers. However, when the ionization energy is increased, the relative heights of the two peaks are significantly different. FIG. 8B shows the spectra at a slightly increased ionization energy of 30%, and FIG. 8C at 35%. Although relative ion abundances are known to fluctuate quite significantly for electrospray ionization, typically on the order of 10%, the differences in peak heights between the two spectra is sufficient, even with a 10% fluctuation, to distinguish the spectra in both of these cases. These spectra are referred to as significantly different from each other. At 30%, there is a greater extent of dissociation of the MS^3 ion (m/z=315.2) of prostaglandin D_2 than of E_2, and at 35%, there is an even greater difference in the relative extents of dissociation.

[0027] The optimal ionization energies for distinguishing between isomers cannot be determined *a priori* but must be identified empirically. In addition, the optimal energy fluctuates with instrument and must be determined separately for each instrument. Preferably, the ionization energy is adjusted until the energy at which the two spectra are most different is determined. This energy can then be used for subsequent experiments.

[0028] It should be noted that the foregoing description is only illustrative of the invention. Various alternatives and modifications can be devised by those skilled in the art without departing from the invention. Accordingly, the present invention is intended to embrace all such alternatives, modifications and variances which fall within the scope of the disclosed invention.